NITROGENASE ACTIVITY DURING THE LIFE CYCLE OF AZOTOBACTER

G. R. Funnell* and Y. T. Tchan*

[Accepted for publication 15th December 1976]

Introduction

The life cycle of Azotobacters has been extensively investigated by Winogradsky (1938), Tchan *et al.* (1962), and Wyss *et al.* (1961). Recently Loperfido and Sadoff (1973) reported on the biosynthesis of macromolecules and nitrogen fixation during cyst germination. A recent comprehensive review has been published by Sadoff (1975) on this subject. However in spite of intensive biochemical investigation of nitrogenase, the details of the status of this enzyme during cyst formation and germination remain incomplete.

Materials and Methods

ORGANISMS

Azotobacter beijerinckii strain VB was used.

MEDIA

N-free medium was used (Winogradsky, 1938). 1% sucrose was added for the growth of vegetative cells. This was replaced by 0·2% n-butanol (autoclaved separately) for cyst formation.

Plates were solidified with 1·5% agar. All media were autoclaved at 101 pKa for 15 minutes.

CHEMICALS

Only A.R. grade chemicals were used.

GROWTH CONDITIONS

Cultures were maintained on Winogradsky's 1% sucrose N-free agar slopes at room temperature. Batch cultures were grown in 300 ml of N-free medium in a 1 litre conical flask on a rotary shaker at 30°C.

OPTICAL DENSITY MEASUREMENT

The optical density (OD) of the cultures was measured on a Unicam SP 600 at a wavelength of 700 nm (1 cm light path), using distilled water.

GROWTH AND NITROGENASE ACTIVITY

Three hundred ml of N-free medium were inoculated with *A. beijerinckii*. At different time intervals (see Fig. 1) duplicated samples were taken for the study of growth and nitrogenase activity.

The nitrogenase activity was expressed :

(i) On a per volume basis: the C_2H_4 peak height obtained from a constant volume of bacteria (0·5 ml) at different OD was used to measure the nitrogen fixing capabilities of a given population during growth.

(ii) On a per cell basis : the C_2H_4 peak height per 0·5 ml of bacterial suspension, adjusted to a pre-determined OD of 0·50, was used to evaluate the nitrogenase activity.

* Department of Microbiology, University of Sydney, New South Wales, 2006.

CYST FORMATION

A culture of *Azotobacter beijerinckii* was harvested, washed in Winogradsky's N-free buffer, and spread over 14 cm diameter Petri dishes containing n-butanol agar. Incubation at 30°C resulted in cyst formation within a week.

CYST GERMINATION

Germination was initiated by suspending cysts in 30 ml of medium containing 1% sucrose. When a combined nitrogen source was used (e.g. urea) it was added up to a suitable final concentration. Incubation was carried out in a Gallenkamp reciprocating shaker held at 30°C and shaken at 100 strokes per minute. Samples were taken for C_2H_2 reduction assays at various time intervals and fixed smears were prepared for monitoring the rate of germination by microscopic examination.

STAINING TECHNIQUES

Violamine stain. The technique of Winogradsky (1938) was used.

Acridine orange stain. A 0·1% stock solution was prepared and stored at 0°C. A 10-fold dilution was used to prepare a wet mount of the cells in the acridine orange solution. A drop of oil was incorporated into the wet mount to prevent free movement of the cells during microscopic examination. Fluorescent microscopy was used to observe the cells.

Vegetative cells were green, whereas the cysts had a green central body and a red coat.

INHIBITION STUDIES

A number of combined nitrogen sources were utilized to examine the possibility of feed-back inhibition of the nitrogenase. Series of concentrated solutions of $(NH_4)_2CO_3$, $NaNO_2$, KNO_3 and urea were prepared; 0·2 ml of a particular nitrogen salt solution was added to 19·8 ml of culture to give a final range of concentration from 0·15 mM to 10 mM. The pH was adjusted to 7·0.

Concentrated solutions of a number of organic combined nitrogen sources were also prepared, (L-glutamate, L-asparagine, glycine, L-lysine and L-serine). One ml of the stock solution was added to 19·0 ml of culture to give a series of concentrations ranging from 3 mM to 20 mM. (The pH was adjusted to 7·0.)

A culture was grown to an OD of 0·8–1·2, at which time appropriate aliquots were added to 150 ml conical flasks. A combined nitrogen source was added to these flasks to the required concentration. Ten minutes were allowed for mixing in a reciprocating water shaker held at 30°C before samples were taken for C_2H_2 reduction assays. The cultures were later harvested and resuspended in an N-free medium and further samples taken for analysis.

ACETYLENE REDUCTION ASSAY

Assay Procedure

Duplicate 12 ml capacity vials with rubber seals, containing 0·5 ml of bacterial suspension in an atmosphere of 80% argon and 20% oxygen were used. 0·8 ml of gas was removed by a tuberculin syringe and replaced by 0·8 cm^3 of C_2H_2. The vials were incubated and gently shaken at 30°C for 3 hours in a Warburg respirometer. Ice was used to stop the reaction. A 200 µl sample of the resultant gas mixture was used in the gas chromatography assay.

The Gas Chromatograph

A Packard gas chromatograph fitted with a flame ionisation detector (Electrometer model 840 : H.V. supply model 838 : dual flow controller model 824 : oven model 802) was used.

Column

A coiled column 45 mm by 3 mm packed with Parapak T was used. The operating conditions were as follows :

column temperature—75°C; inlet temperature—70°C ; detector temperature —100°C ; carrier gas flow rate—100 cm^3/min ; H_2 flow rate—60 cm^3/min ; air flow rate—450 cm^3/min.

The acetylene and ethylene peaks were separated within 90 seconds. The C_2H_4 peak height (in mm) was used to measure the nitrogenase activity.

Results and Discussion

Nitrogenase activity in vegetative cells and during cyst formation

The nitrogenase activity of vegetative cells was measured during a growth cycle (Fig. 1). The activity of the enzyme per unit of volume increased during the first 20–22 hours until an optical density (OD) of 1·5 was reached. After this initial period, the activity remained constant for the next 10 hours and then decreased quickly to nil in the following 8 hours. The cell division did not

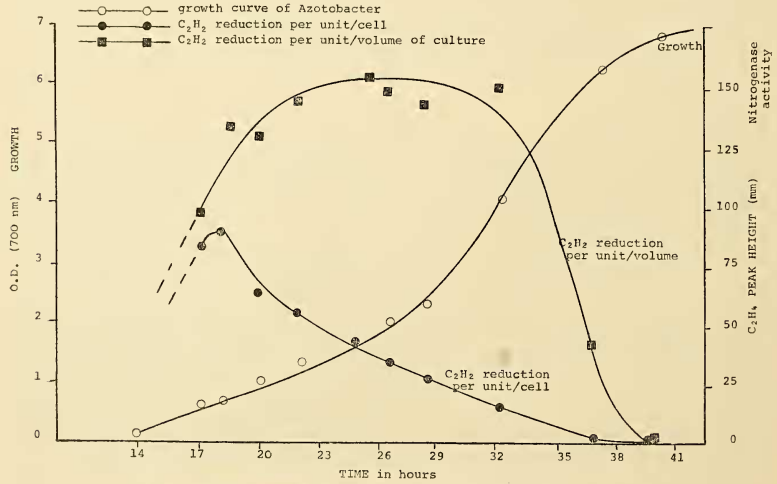

Fig. 1. Nitrogenase activity during the growth of Azotobacter.

follow the same pattern. The logarithmic growth continued until the stationary phase was reached at 40–42 hours. It was apparent that during the 10 hour period (from 22 hours to 32 hours) the total activity of nitrogenase per volume remained constant in spite of increase in cell density. Either there is a repression of enzyme synthesis or a reduction of its activity during logarithmic growth of the culture. The enzymic activity decreased further towards the end of the exponential phase and eventually ceased when the stationary phase was reached.

The sudden drop in activity approaching the stationary phase of growth suggested the possibility of either destruction or inactivation of the enzyme. When a few grains of sucrose were introduced to the reaction vial containing a culture which had recently reached the stationary phase, the activity was

rapidly restored. This indicated that a shortage of energy supply was responsible for the drastic reduction in activity.

In order to ascertain if the inactive nitrogenase could persist in the cell prior to its encystment, a culture which had ceased its nitrogenase activity for 72 hours was used. Two 30 ml aliquots were taken and treated as follows: (a) control, (b) with an addition of 1% sucrose. After three hours of incubation only treatment (b) gave some small nitrogenase activity. These results demonstrated that nitrogenase activity was practically nil and could not be restored rapidly.

From the above experiment, it can be inferred that the cells undergoing encystment were almost completely depleted of active nitrogenase.

NITROGENASE DURING THE GERMINATION OF CYSTS

If the cysts were depleted of active nitrogenase prior to their formation, it would be important to investigate the activity of this enzyme during the germination of the cyst.

The germination of cysts in N-free sucrose medium was followed microscopically using orange acridine and violamine staining. Samples were also taken at regular intervals to follow changes in nitrogenase activity. A parallel experiment with a medium containing combined nitrogen (200 ppm of urea) was performed (see Table 1).

TABLE 1

Nitrogenase activity and germination of cysts in nitrogen-free and in urea media

Time (hours)	Nitrogenase activity	Germination %		Total % of Germination
	(The activity % using total germinated as 100%)	Free cell	Displacement of central body	
Nitrogen-free medium				
0	0	0	0	0
2·5	23	0	3·0	3·0
9·25	100	38	62	100
Medium with 200 ppm of urea				
2	0	36	5	41
6	0	100	0	100

In the presence of combined nitrogen, cyst germination proceeded at a much greater rate. As urea is a well known repressor of nitrogenase, it demonstrated that the activity of this enzyme was not essential in cyst germination when combined nitrogen was available. There was a considerable discrepancy between the nitrogenase activity and cyst germination. After two hours of incubation, very few cysts showed sign of germination (only 3·0% of cells showed any displacement of the central body) but the nitrogenase activity had already reached 23% of that of a fully germinated cyst suspension. This suggested that nitrogenase activity had developed prior to the cytological evidence of germination. Also after the initial lag period (one hour) the nitrogenase activity increased linearly with time until all the cysts had germinated (Fig. 2).

The inhibition of nitrogenase by urea did not exclude the possibility of synthesis of precursors of nitrogenase nor accumulation of other factors essential for nitrogenase activity. After four hours of incubation in a medium containing 200 ppm of urea, cysts were harvested and washed in Winogradsky's mineral

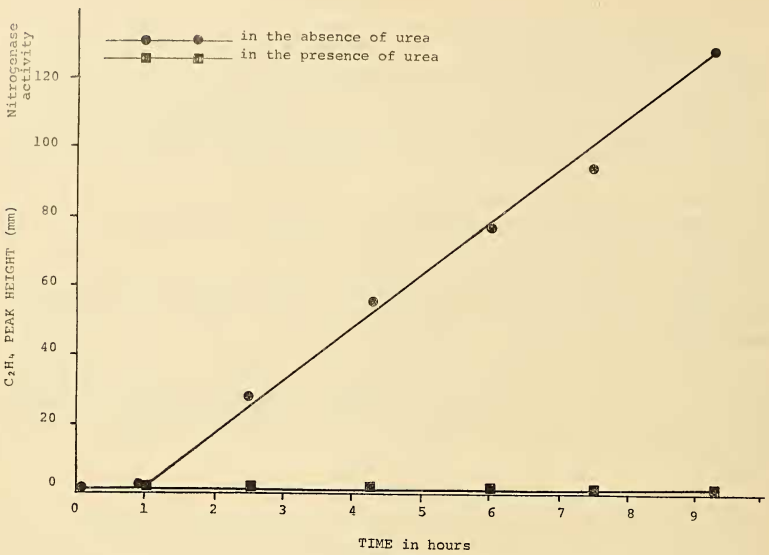

Fig. 2. Nitrogenase activity in the presence and absence of urea.

solution and resuspended in N-free medium. Samples were taken regularly for nitrogenase activity assays. A control experiment was performed whereby cysts were germinated initially in an N-free medium for three hours, followed by the addition of 200 ppm of urea and a further incubation of one hour. The cysts were later harvested, washed and resuspended in an N-free medium. Samples were also taken at regular intervals during this experiment for nitrogenase activity assays (Fig. 3).

It was evident that after a short lag period the nitrogenase activity increased linearly. The activity was completely inhibited upon addition of urea. This inhibition was entirely reversible since the activity was restored upon removal of the urea. There was no nitrogenase activity in cysts germinated in the presence of urea. Upon removal of urea, an immediate increase of nitrogenase activity occurred at a rate comparable to that of N-free cultures. However, the disappearance of the lag period suggested that initial activation of nitrogenase had occurred during germination in the presence of urea.

INFLUENCE OF PH ON NITROGENASE ACTIVITY

Some supporting evidence for the activity of nitrogenase in the cyst prior to its germination was obtained in an acid environment. A comparative study of the nitrogenase activities of vegetative cells and germinating cysts was carried out. It was clear that the nitrogenase activity of vegetative cells decreased with a lowering of pH. All activity ceased at a pH of 5·6. With cysts, after a short lag period nitrogenase activity was detected in a medium at a pH as low as 4·3 (see Table 2).

TABLE 2

Influence of pH on nitrogenase activity of germinating cyst and vegetative cells

pH		9·4	8·0	7·6	7·0	6·5	6·2	5·6	5·4	5·0	4·3
Percentage of nitrogenase activity	Cyst	100	100	—	—	100	—	—	70	—	70
	Veg. cell	—	100	94	81	75	50	0	—	0	—

There is no reason to believe that the vegetative cell directly produced from a cyst would have better tolerance to pH than a normal cell. Therefore when the cyst coat is ruptured the nitrogenase activity of the emergent cell

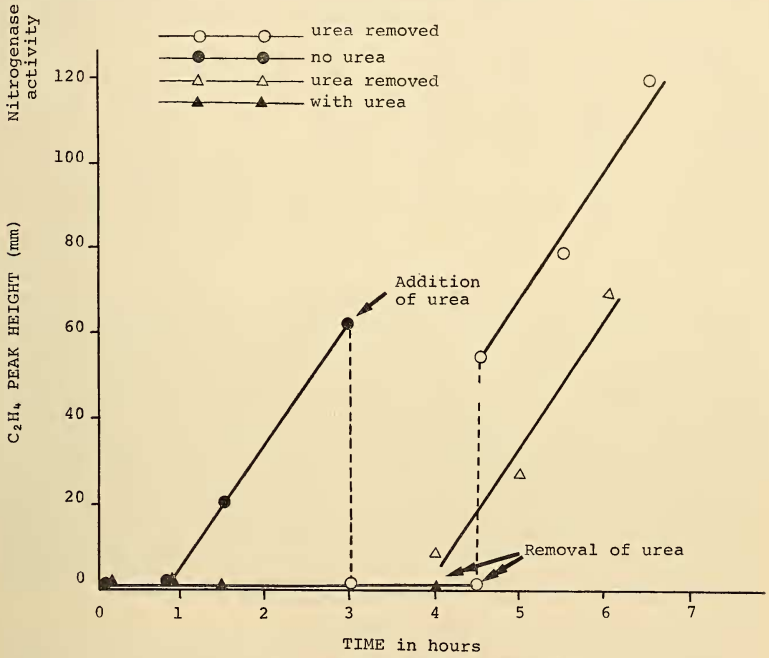

Fig. 3. Influence of urea on nitrogenase activity.

would be impaired in an acid environment. The measured activity of nitrogenase from a germinating cyst would most likely come from the germinating cell prior to the rupture of its cyst coat.

INHIBITION OF NITROGENASE ACTIVITY IN VEGETATIVE CELLS

The C_2H_2 reduction per unit of cells in Fig. 1 showed a constant reduction in nitrogenase activity during the logarithmic growth of the culture. Such a reduction could not be attributed to a lack of energy supplies since the synthesis of cell mass still progressed at a logarithmic rate. It was suggested that the

possibility of a leakage of nitrogenous compounds may be responsible for such inhibition. The influence of the following chemicals on nitrogenase activity was tested: NH_3, urea, NO_2, NO_3, L-serine, L-lysine, glycine, L-asparagine and L-glutamine. In all cases the pH was carefully adjusted and maintained at neutrality.

Only urea and NH_3 would inhibit nitrogenase activity at a low concentration (0·15 mM) (see Table 3). It was not likely that the accumulation of nitrogenous compounds in an actively growing culture could exceed 0·5 mM and therefore the hypothesis of nitrogenase inhibition by organic molecules could not be substantiated. No attempt was made to explain this phenomenon (see Table 3).

Table 3

Influence of nitrogenous compounds on nitrogenase activity of vegetative cells (percentage of activity)

Concentration mM	0	0·15	0·50	1	2	3	6	9	14	20
Urea	100	94	75	60	30	10	—	10	—	—
NH_3	100	87	70	61	25	5	—	2	—	—
NO_2	100	118	130	87	25	3	—	1	—	—
NO_3	100	120	86	50	24	22	—	21	—	—
L-serine ..	100	—	—	—	—	97	88	86	91	—
L-lysine ..	100	—	—	—	—	98	89	100	98	—
Glycine ..	100	—	—	—	—	140	138	135	125	—
L-asparagine ..	100	—	—	—	—	135	136	123	140	—
L-glutamine ..	100	—	—	—	—	108	110	114	136	144

Our experiment also demonstrated that cessation of nitrogenase activity in cysts was not due to the repression of enzyme activity. Therefore one of the processes of cyst germination was the initiation of nitrogenase activity if the germination occurred in a nitrogen free environment. Our data differed from the findings of Loperfido and Sadoff (1973) which stated that nitrogen fixation started when the outgrowth (defined by these authors as the time at which cysts rupture and release vegetative cells) occurred, which suggests that nitrogen fixation started when the cyst was transformed into a vegetative cell. Our results indicated that the nitrogenase was active prior to cytological evidence of germination and suggested that it was not necessary to reach the vegetative stage of development to initiate nitrogen fixation. Furthermore in an adverse environment (acid pH) the cyst coat will protect the enzyme from its inactivation by hydrogen ions. It is also interesting to note that in spite of the presence of a powerful repressor (urea) the lag period of nitrogenase activity observed in germinating cysts in N-free medium was eliminated.

Acknowledgement

The authors wish to acknowledge the helpful criticism given by Professor J. M. Vincent, Dr. K. Y. Cho and Dr. I. R. Kennedy of the University of Sydney, during the preparation of the manuscript.

References

Loperfido, B., and Sadoff, H. L., 1973.—Germination of *Azotobacter vinelandii* cysts ; sequence of macromolecular synthesis and nitrogen fixation. *J. Bacteriol.*, 113 : 841–846.

Sadoff, H. L., 1975.—Encystment and germination in *Azotobacter vinelandii*. *Bacteriol. Rev.*, 516–536.

Tchan, Y. T., Birch-Anderson, A., and Jensen, H. L., 1962.—The ultra structure of vegetative cells and cysts of *Azotobacter chroococcum*. *Arch. Mikrobiol.*, 13 : 50–66.

Winogradsky, S., 1938.—Sur la morphologie et l'écologie des Azotobacter. *Ann. Inst. Past.*, 60 : 351–400.

Wyss, O., Neumann, M. G., and Socolofsky, M. D., 1961.—Development and germination of the Azotobacter cyst. *J. Biophys. Biochem. Cytol.*, 10 : 555–565.